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ORIGINAL PAPER

Expression analysis of *ThGLP*, a new germin-like protein gene, in *Tamarix hispida*

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Abstract: Germin and Germin-like protein (GLP) have various proposed roles in plant developmental stages and stress- related processes. A novel *GLP* cDNA clone was isolated from a cDNA library of *Tamarix hispida*. *ThGLP*, coded 225aa, possesses conserved motif of plant germin and Germin-like protein. ThGLP belongs to true germin subfamily through phylogenetic analyses. Gene expression profiles in roots and leaves were evaluated using real-time quantitative RT-PCR. The results show that the gene was highly induced by drought, salt, low temperature, $CdCl_2$ and abscisic acid treatments. Our results demonstrate that the *ThGLP* gene is expressed in leaves and roots, is involved in different abiotic stress responses and controlled by an ABA-dependent signaling pathway.

Keywords: germin-like protein (GLP); *Tamarix hispida*; abiotic stress; gene expression

Introduction

Germins and germin-like proteins (GLP) constitute a large family of proteins ubiquitously distributed in plant. They are part of a superfamily of proteins together with seed storage globulins and sucrose-binding proteins. Although the overall amino acid sequence conservation of the cupins is quite low, all these proteins have a predicted β-barrel structure and histidine- containing motifs in common (Requena et al. 1999; Dunwell et al. 2008).

Germins have an oxalate oxidase activity, which plays a significant role in plant development and defense (Lane 2000). Germin-like proteins (GLPs) exhibit sequence and structural similarity with germins but mostly lack oxalate oxidase activity. Furthermore, GLP and GLPs exhibit a broad range of diversity in their occurrence, found in leaves, cotyledons, stems, roots, flowers and seeds (Membré et al. 2000; Fan et al. 2005; Yin et al. 2009). In addition, Germins and GLPs are thought to play a significant role during zygotic and somatic embryogenesis (Caliskan et al. 2004; Neutelings et al. 1998; Mathieu et al. 2003), salt stress (Berna et al. 1999), drought stress (Ke et al. 2009), pathogen elicitation (Jøhnk et al. 2005; Godfrey et al. 2007; Manosalva et al. 2009), and heavy metal stress (Zhou et al. 2009), etc..

Tamarix hispida is a shrub or small tree exhibiting tolerance to various stresses, including salt, drought and low temperatures. In the present study, a gene encoding germin-like protein (named *ThGLP*) was isolated from a *Tamarix hispida* NaCl-stress root cDNA library. To better understand the possible roles of *ThGLP* in defense against abiotic stress, a time course expression analysis of *ThGLP* gene was performed. We studied the expression of *ThGLP* in response to drought, salt, low-temperature, $CdCl_2$ and abscisic acid (ABA) treatments in roots, stems and leaves of *T. hispida*. Characterization and cloning of the gene encoding GLP has facilitated a better understanding of their regulation and raised their potential of biotechnological application.

Materials and methods

Plant materials and treatments

Tamarix hispida seedlings were planted in a mixture of turfy peat and sand (2:1 v/v) in a greenhouse with 75% relative humidity and a constant temperature of 24°C. In order to detect induction of *ThGLP* under different abiotic stress treatments, 3-month-old *T. hispida* seedlings were treated with 400 mM NaCl, 4ºC, 20% (w/v) PEG6000, 100 μM ABA or 150 μM $CdCl_2$. Leaf and root tissues of the seedlings were harvested at 0, 6, 24, 48 and 72 h

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after each treatment and immediately frozen in liquid nitrogen and stored at -80°C until RNA preparation.

Phylogenetic analysis

Molecular weight (MW) and isoelectric point (pI) were computed with the ExPASy Compute pI/Mw tool at (http://www.expasy.org/tools/protparam.html). Signal peptide prediction were completed using the Signal peptide tool (http://www.cbs. dtu.dk /services/SignalP/). Special function prosite was predicted using prosite software (http://cn.expasy.org/prosite). All GLPs sequences were aligned using ClustalX and were subjected to phylogenetic analysis by conducting a phylogenetic tree reconstruction using MEGA4.

Sample preparation for real time quantitative RT-PCR

Total RNA was extracted from leaf and root tissues of *T. hispida* plants using a cetyl trimethyl ammonium bromide (CTAB) method. The extracted RNA was then treated with RNase-free DNase I (Promega) to remove any residual DNA. Total RNA (1μg) then was reverse-transcribed to cDNA using a 20μL volume of an oligodeoxythymidine primer following the PrimeScript^{TM} RT reagent Kit (TaKaRa) protocol. The 1μL synthesized cDNAs were diluted to 10 μL with sterile water and used as the template for real-time PCR.

Table 1 The primer sequences used in internal references and GLP genes for qRT-PCR

Accession No	Gene name	Forward primer nucleotide sequence (5'-3')	Reverse primer nucleotide sequence (5'-3')
EG971352	β-*actin*	AAACAATGGCTGATGCTG	ACAATACCGTGCTCAATAGG
EH050602	α-*tublin*	CACCCACCGTTGTTCCAG	ACCGTCGTCATCTTCACC
EH052343	β-*tublin*	GGAAGCCATAGAAAGACC	CAACAAATGTGGGATGCT
EF416283	*18s rRNA*	GTAGTTGGACCTTGGGGTGG	CATTACTCCGATCCCGAAAGCC
	ThGLP	CCATCACACTGTGCTGATCC	GGTTAAGCCCAGCAACAGC

Real-time quantitative RT-PCR

Real-time RT-PCR was carried out in an Opticon^{TM}2 machine (Biorad, Hercules, Calif) using a real-time PCR MIX Kit (SYBR Green as the fluorescent dye, TOYOBO). Primers chosen for real-time RT-PCR are given in Table 1. The α-*tublin* (EH050602), β-*tublin* (EH052343), β-*actin* (EG971352) and *18s rRNA* (EF416283) genes were used as internal references to normalize the amount of total RNA present in each reaction. PCR was performed in a total volume of 20 μL, containing 10 μL of SYBR Green Realtime PCR Master Mix, 0.5 μM of each forward and reverse primer, and 2 μL of cDNA template. The amplification was completed using the following cycling parameters: 94°C for 30 s followed by 45 cycles of 94°C for 12 s, 58°C for 30 s, 72°C for 45 s and 1 s at 81°C for plate reading. A melting curve was generated for each sample at the end of each run to serve as an assessment of purity for the amplified products. Three independent experiments (biological replicates) of the real-time PCR were carried out to ensure the reproducibility of results. After the reactions were completed, samples were also electrophoresed in agarose gels to verify amplification of the target fragments. The Clone expression levels were calculated from the threshold cycle according to the 2 (-Delta Delta Ct) method (Livak and Schmittgen 2001).

Result analysis

Characteristics of *ThGLP*

The full-length cDNA of *ThGLP* was primarily isolated from a *T. hispida* NaCl-stress root cDNA library (Li et al. 2009). The cDNA is 878 bp with a 5′-UTR of 80 bp, an open reading frame of 675 bp, and a 3′-UTR of 123 bp. The amino acid sequence deduced from the open reading frame revealed that ThGLP encodes a protein of 225 amino acids with a calculated *MW* of 23.8 kDa and a theoretical *pI* of 5.89. An N-terminal signaling peptide showed that the most likely cleavage site is between position 28 and 29 (SHC-AD), suggesting a role in cell wall function or defense against invading pathogens (Park et al. 2004).

Alignments of the amino acid sequence of *T. hispida* with other 10 sequences. ThGLP possesses the characteristics common to plant germins and GLPs (Lane et al. 1991; Membré et al. 2000): three highly conserved oligopeptides (boxes A, B, and C), two Cys residues (Cys-38 and Cys-53) known to form disulfide bonds, and three His residues (His-115, His-117, and His-161) (Fig. 1) involving in binding a metal ion. Moreover, A motif search revealed one potential *N*-glycosylation sites (Asn-139) (Jaikaran et al. 1990) and five *N-myristoylation* site (73–78: GNtdNV; 86–91: GNvvTF; 98–103: GIalNR; 171–176: GLilTA; 219–224: GGlaNA). These findings showed that ThGLP belongs to the GLP family. However, the function of ThGLP remained to be elucidated.

ThGLP is similar to germins and GLPs

Phylogenetic analyses classified germins and GLPs into five subfamilies (Carter and Thornburg 1999; 2000; Druka et al. 2002) or three subfamilies (Khuri et al. 2001) depending on how the query was designed. In this study, we prefer the phylogeny analyzed by Carter and Thornburg (1999, 2000) because the entire GLP sequence was used in the analysis. The GenBank contains at least 80 full-length or near full-length sequences encoding germin-like proteins. Eighty-four GLP sequences (see Fig. 2) are identified five phylogenetic clades. ThGLP is a new member of true germin subfamily and genetic distance is so near with subfamily 2. The true germin clade contains most of the wheat and

barley germins along with one Arabidopsis, and one rice GLP (total of 8 sequences). We also identify a clade of ten sequences, referred to as the gymnosperm GLPs. All of the remaining plant GLPs fall into three families: subfamily 1 (27 members), subfamily 2 (17 members), and subfamily 3 (20 members).

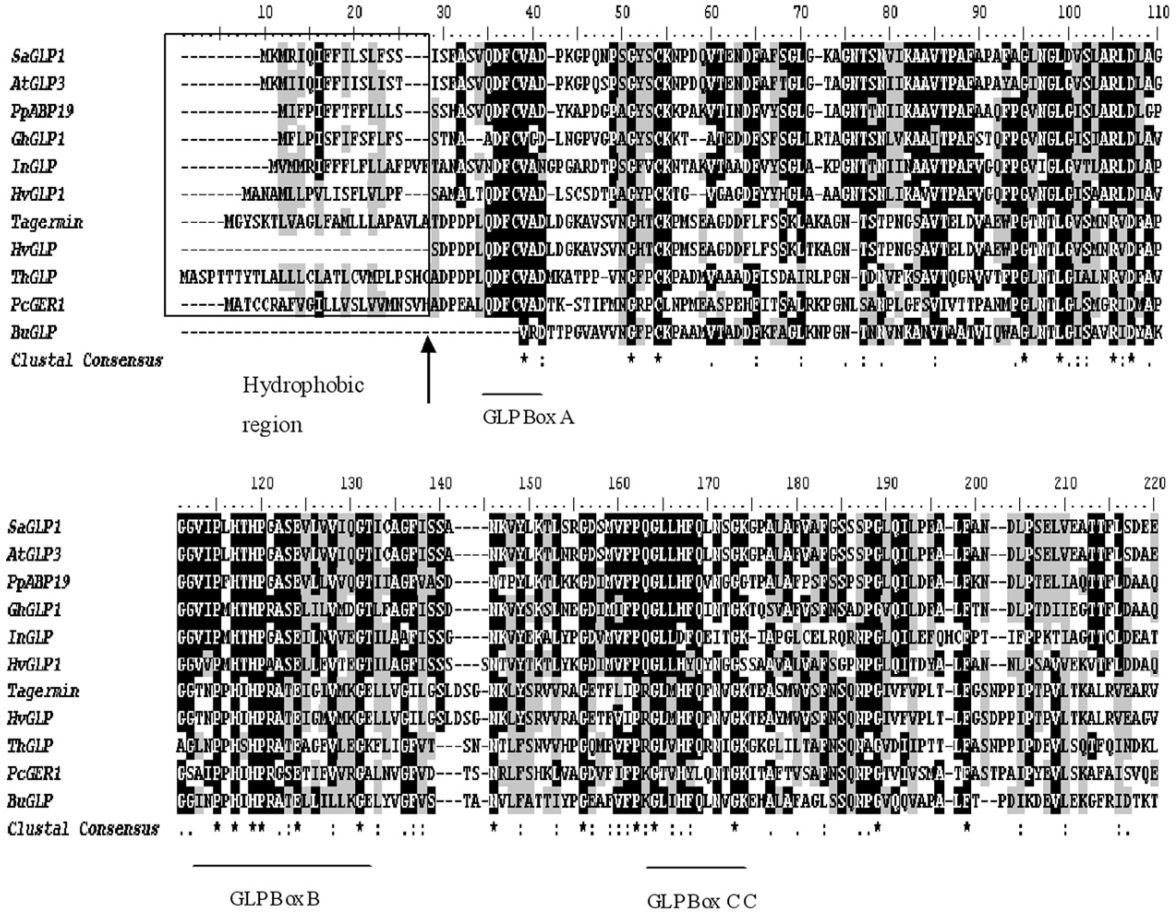

Fig.1 Multiple alignment of GLPs. Amino acid sequences deduced from *Sinapis alba* (SaGLP1, CAA59257), *Prunus persica* (PpABP19, AAD00295), *Hordeum vulgare* (HvGLP1, Y15962), wheat (Ta-germin OXO, A33268), *Barbula unguiculata* (BuGLP, AB028454), *Arabidopsis thaliana* (AtGLP3, NP_197563), *Gossypium hirsutum* (GhGLP1, AAF21988), *Ipomoea nil* (InGLP, BAA08266), *Hordeum vulgare* (HvGLP, AAA32959), *Pinus caribaea* (PcGER1, AAC99473)are aligned. Conserved sequences are highlighted. X Any hydrophobic amino acid; GLP Box A, B, C conserved amino acids of the germin/GLP

The wheat and barley germins (true germin clade) have oxalate oxidase activity and barley germin also has superoxide dismutase activity (see Table 2). Outside of the true germin clade, oxalate oxidase activity has not been reported. The finding that members of three separate clades (Gymnosperm GLP Subfamily, subfamilies 1 and 2) of the phylogenetic tree contain GLPs with superoxide dismutase activity implies that superoxide dismutase activity maybe widespread throughout this protein family.

Further in silico analysis indicated that *ThGLP* maybe has oxalate oxidase activity and superoxide dismutase activity. It is therefore likely that this gene plays an important role in the response to abiotic stress. To further investigate the function of the gene, the expression levels of *ThGLP* under five treatments were investigated.

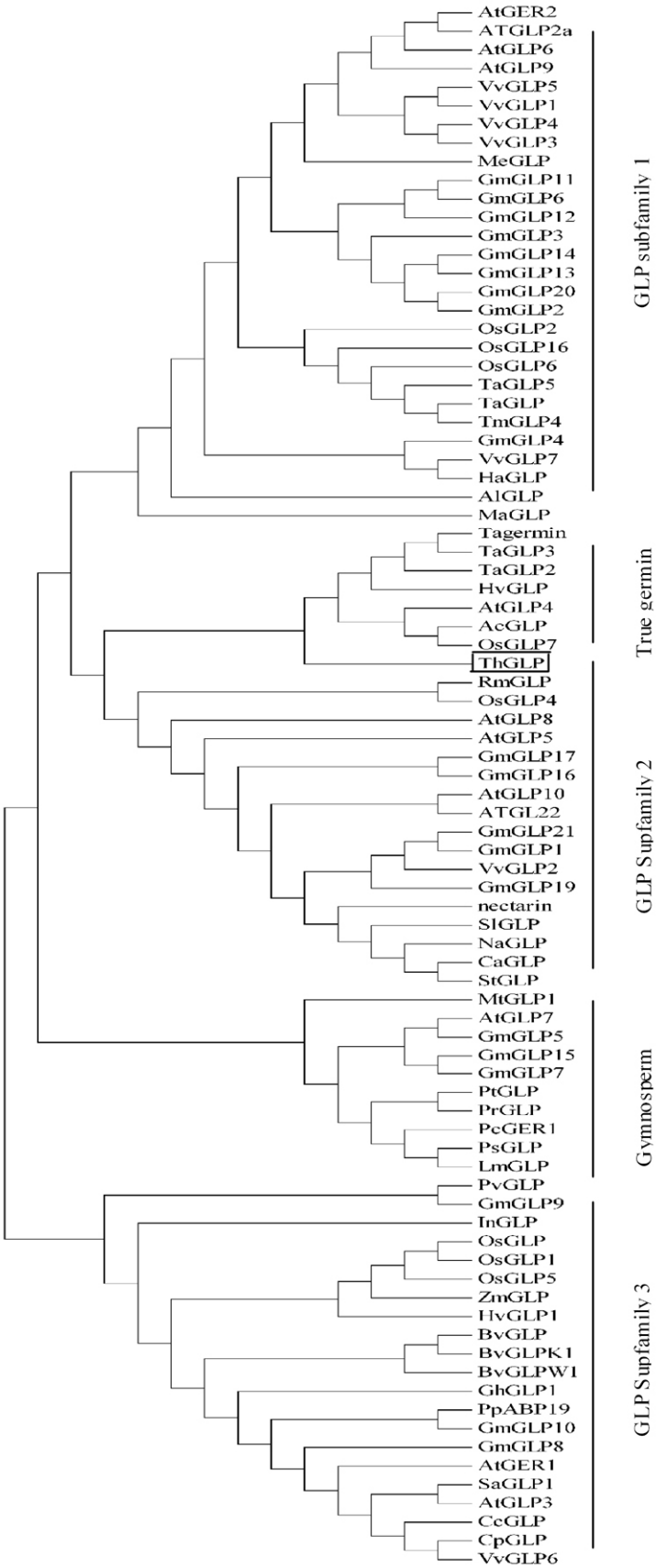

Fig.2. Phylogenetic analysis of 84 germin-like protein sequences.

Ta germin, AAA34270; HvGLP, AAA32959; PcGER1,AAC99473; PpABP19, AAD00295; SaGlp1, CAA59257; GhGLP1, AAF21988; PsGLP, AAL79929; AtGER2, NP_001119334; AtGLP4, NP_564067; AtGLP10, NP_974477; AtGLP7, NP_563870; AtGLP6, NP_568562; AtGER2, NP_198735; AtGLP8, NP_187244; AtGLP10, NP_191761; AtGLP5, NP_172427; AtGER3, NP_197563; AtGER1, NP_177405; AtGLP9, NP_193199; AlGLP, BAA78563; TaGLP ,CAB55559; OsGLP, BAD09958; PsGLP, CAB65370; PvGLP ,CAB77393; CaGLP, CAI56441; ZmGLP, AAQ95582; PcGER1,AAC99473; NaGLP, AAR97545; RmGLP, BAG75123; LmGLP, AAY57280; MaGLP, AAL05886; HaGLP,ABW89137; SlGLP, BAA25197; BvGLP, CAK22420; CcGLP, ABG76199; AcGLP, AAM28275; StGLP, AAC78470; PrGLP, AAC05146; InGLP, BAA08266; VvGLP7, ABL60876; VvGLP6, ABL60875; VvGLP5, ABL60874; VvGLP4, ABL60873; VvGLP1, ABL60872; VvGLP2, ABH09468; AtGLP2a, NP_198732; OsGLP1, BAB17848; TmGLP4, AAT67049; HvGLP1, CAA75907; MtGLP, AAO32795; OsGLP7, AAC25777; OsGLP 16, AAB97470; OsGLP21, ACL14493; GmGLP1,ACG69497; GmGLP20, ACG69496; GmGLP19, ACG69495; GmGLP18, ACG69494; GmGLP17, ACG69493; GmGLP16, ACG69492; GmGLP15, ACG69491; GmGLP14, ACG69490; GmGLP13, ACG69489; GmGLP12, ACG69488;

Table 2. Proposed enzymatic activities or physiological functions of germins and germin-like proteins (GLPs).

Subfamily	Members	Protein accession No	Tissue or Developmental stage	Enzymatic activities or Suggested functions	References
GLP Subfamily1	VvGLP3	ABL60874	cell wall	SOD	Godfrey et al. 2007
GLP Subfamily 2	NaGLP	AAR97545	leaves	SOD	Lou et al.2006
	RmGLP	BAG75123	apoplast	SOD	Kondo et al.2008
	Nectarin I	AAK95664	floral nectar	SOD	Carter et al.2003
GLP Subfamily 3	PpABP19	AAD00295	shoot apex	Putative ABP	Ohmiya et al. 1998
	AtGLP3	CAA73213	leaf and flower	Putative receptor	Membre´ et al. 2000
	HvGLP1	CAA75907	leaf	AGPPase	Vallelian-Bindschedler et al. 1998
	GhGLP1	AAF21988	elongating, fiber cell	Cell expansion	Kim et al. 2004
True germin subfamily	Wheat germin	AAA34270	germinating, embryo	OxO	Lane et al. 1991
	Barley germin	AAA32959	seedling	OxO, Mn-SOD	Woo et al. 2000
	AtGLP4	NP_564067	golgi	cell growth	Yin et al. 2009
Gymnosperm GLP Subfamily	PsGER1	CAB65370	meristematic zone, epidermis	SOD	Gucciardo et al. 2007
	LmGLP	AAY57280	somaticembryogenesis, vascular procambium and xylem	SOD	Mathieu et al. 2006

Expression patterns of *ThGLP* under different abiotic stress conditions

The mRNA levels of *ThGLP* under the stress of high salt, drought, low temperature, heave metal, and exogenous ABA treatment were detected with Real-time quantitative RT-PCR (Fig. 3a-f). Under high-salt conditions, the gene had different expression patterns in leaf tissues and root tissues (Fig. 3a). In leaf tissues, the mRNA level of *ThGLP* was up-regulated transiently at 6 h of stress (4-fold), then rapidly decreased, and reached their lowest expression level at 24 h of stress (decreased 80% compared with 0 h). In contract, the expression of *ThGLP* in root was also highly induced and reached a peak at 48 h, with an induction level from 1.44- to 10.51-fold. *ThGLP* gene exhibited different expression patterns under drought stress. In leave tissue, the gene was induced after treated with drought treatment. At 24 and 72 h, the expression was over 8-fold higher than in the control sample (Fig. 2b). In contract, the expression of the gene in roots was down-regulated during the PEG stress, the expression levels at 24 h, 48 h and 72 h of stress decreased 80%–90% compared with 0 h. Under low temperature treatment (4ºC), the expression level of *ThGLP* in leaf tissues showed rapid up-regulation during the cold treatment, reached a peak at 6 h of stress treatment (12-fold), with an induction level from 2.5- to 12-fold (Fig. 3c). While in roots, the expression profile of *ThGLP* was similar to that of drought treatment, only the expression level of the gene at 48 h was higher than that of 0 h, at other time point the expression levels of the gene were lower compared with 0 h. *ThGLP* exhibited gradually induction by $CdCl_2$ in leave, and the highest expression level was at 24 h and 48 h of stress, the expression of *ThGLP* in leave increased nearly 5-fold and 10-fold, compared with the 0 h level (Fig. 3d). In the roots, the expression patterns of the gene exhibited increase, decrease, decrease. The expression level was rapidly increased at 24 h treatment (8-fold), reached the lowest level at 48 h, then restored. During the exogenous ABA treatment, the expression level of *ThGLP* was progressively up-regulated by ABA in leave, and the highest expression level was at 72 h of treatment (8- fold) (Fig. 3e). The expression pattern of the gene in roots was similar to that of $CdCl_2$ treatment. The highest expression level appeared at 24 h of treatment, accordingly the lowest at 72 h of the treatment. belongs to true germin subfamily, which maybe has the characteristic features of the superfamily.

Discussion and Conclusion

We identified a gene, *ThGLP*, its deduced protein sequence possesses of the characteristics of germin and germin-like protein family, which belongs to the cupin superfamily (Woo et al. 2000). Germins and GLPs have falled into five groups: true germin subfamily; gymnosperm GLP subfamily; GLP subfamily 1; GLP subfamily 2; and GLP subfamily 3. Germin-like proteins possess of diversified functions. Oxalate oxidase activity has been known to be associated with only the true germin subfamily (Table 1). On the other hand, superoxide dismutase (SOD) activity has been found in GLPs of subfamilies 1, 2 and Gymnosperm GLP Subfamily. Members of GLP subfamily 3 are expressed specifically in leaves; and their mRNA levels undergo circadian oscillations (Staiger et al.1999). Through phylogenetic analysis, ThGLP

Plant exposure to environmental abiotic stress conditions such as salt, drought, cold and heavy metal pollutants can lead to an excess accumulation of reactive oxygen species (ROS) in cells. This build-up of ROS results in toxicity, which can non-specifically damage many cellular components. Superoxide dismutase and oxalate oxidase have been shown to be capable of oxygen reduction (Vuletic and Sukalovic. 2000). In order to determine whether *ThGLP* was induced by abiotic stresses, the RNA levels of the gene were detected under five stress treatment using real-time PCR. In the study, expression of GLP genes has been shown to be up-regulated evidently in roots by salt stress in *T. hispida*, this result is accordant with in barley and *M. crystallinum* (Michalowski et al.1992), NaCl delayed barley germination and increased the amount of germin polypeptides and mRNAs in youngseedling roots (Hurkman et al. 1996; Nakata et

al. 2004; Dani et al. 2005). *AlGLP* transcripts were most abundant in calli, and present in roots, but were not detected in stems or leaves under salt stress. But In roots, the expression of *AlGLP* was inhibited by salt stress (Tabuchi et al. 2003). Under drought and cold stress, the expression levels of *ThGLP* were up-regulated in leaves, but in roots, the expression level was obviously down-regulated compared with 0 h. However, in barley, it has been reported that the transcription of the GLP in roots (68 ESTs) was more than the transcription in shoots (56 ESTs) either stressed by a cold or a dehydration treatment (Druka et al. 2002). Germin accumulation is stimulated by heavy metal ions copper, cobalt and cadmium in wheat seedlings (Patnaik et al.2001). The RNA of *ThGLP* was accumulated in leaves and roots during the time of $CdCl_2$ treatment. The set of results indicates that *ThGLP* was induced by abiotic stresses, and *ThGLP* maybe contribute to resist the toxicity resulted from abiotic stresses. On the other hand, the response of *ThGLP* to abiotic stresses was similar with wheat germin. Therefore, the function of *ThGLP* is likely similar with wheat germin, maybe have oxalate oxidase or superoxide dismutase activities.

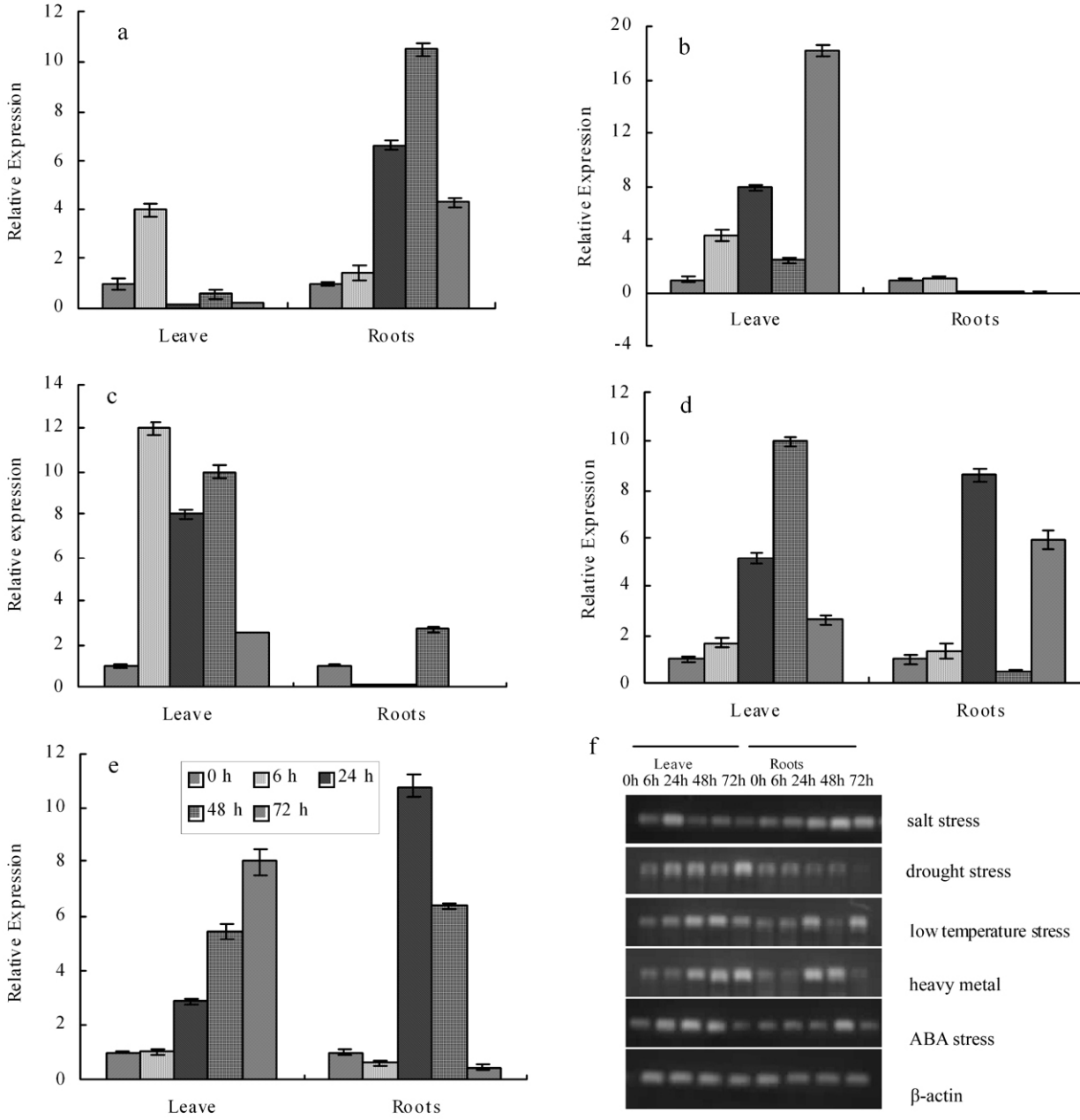

Fig. 3 *ThGLP* gene expression levels under different abiotic stresses assayed using Real-time quantitative RT-PCR. a: salt stress (400 mM NaCl); b: drought stress (20%PEG6000); c: Low temperature stress (4℃); d: heavy metal (150 μM $CdCl_2$); e: ABA stress(100 μM); f: gal images of RT-PCR.

In summary, we obtained a full-length *ThGLP* gene from a *T. hispida* NaCl-stress root cDNA library. The gene belongs to true germin subfamily using phylogeny analysis. The expression profiles of *ThGLP* were constructed response to different abiotic

and ABA treatments stress, the expression analyses demonstrated that the *ThGLP* has involved in an abiotic stress response and been regulated by ABA-dependent signaling pathways.

Acknowledgments

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